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AMILORIDE-INHIBITED Na^+ UPTAKE INTO TOAD BLADDER MICROSOMES IS Na^+-H^+ EXCHANGE

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Amiloride-inhibited Na^+ transport into toad urinary bladder microsomes is sensitive to a pH gradient across the vesicular membrane. The magnitude of the gradient was measured directly with acridine orange. Also Na^+ could stimulate amiloride-sensitive proton efflux from the microsomes. These results indicated that the transport process was Na^+-H^+ exchange.

Microsomal vesicles capable of amiloride-inhibited Na^+ transport [1] can be formed from the urinary bladder of the tropical toad, *Bufo marinus*. Since the amiloride concentration required for inhibition of Na^+ transport into these microsomes was rather high ($> 10^{-3}$ M), it seemed likely that the protein responsible for this transport was capable of Na^+-H^+ exchange rather than electrogenic, conductive Na^+ movement. Most studies of amiloride-inhibited Na^+ conductance across apical membranes of tight epithelia have shown inhibitory effects of as little as 10^{-6} M amiloride [2–5]. The amount of amiloride required to inhibit Na^+-H^+ exchange has generally been much higher (10^{-3} M) [6–10]. Therefore, we examined the sensitivity to pH gradients of the amiloride-inhibited Na^+ transport into toad bladder microsomes. We have also looked at the ability of Na^+ transport to produce pH gradients across the microsomal membranes. Our results indicate that amiloride-inhibited Na^+ transport into toad bladder microsomes is Na^+-H^+ exchange.

Toad bladder microsomes were formed and Na^+ transport into these microsomes was measured as previously described [1]. We have not attempted to determine the specific orientation of the proteins in these vesicles. To demonstrate pH gradients in the microsomes, acridine orange uptake into the microsomes was measured by a modification of existing procedures [11,12]. The microsomes were pre-incubated for 40 min at 20°C with acridine orange (30 μM) and either pH 6.0 or pH 8.0 buffer solutions, (composition detailed in the legend of Table I) in a total volume of 80 μl and then incubated for 2 min at 20°C with either pH 6.0 or pH 8.0 buffer solution with or without amiloride methanesulfonate, with or without sodium methanesulfonate in a total volume of 0.25 ml and then applied to Dowex columns [1] to remove extravesicular acridine orange. The columns were eluted with 1 ml sucrose solution (0.25 M) at 0°C, the effluents, which contained only the intravesicular acridine orange, were sonicated for 1 min and the fluorescence of the effluents measured at an excitation wavelength of 470 nm and an emission wavelength of 550 nm. As a control, microsomes

Abbreviations: SITS, 4-acetamido-4′-isothiocyanostilbene 2,2′-disulfonate; Mes, 4-morpholineethanesulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Hepps, 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid.

were incubated with buffer in the absence of acridine orange or acridine orange incubated in the absence of microsomes, and the incubation mixtures applied to Dowex columns. The fluorescence of the eluants was found to be less than 5% of the fluorescence observed when acridine orange was incubated with microsomes as described above.

Amiloride-sensitive Na^+ transport into toad bladder microsomes was stimulated when there was a pH gradient with more acidic pH inside the microsomes. To show that we could establish a pH gradient from the inside of the vesicles to outside, we measured acridine orange uptake by the method outlined above. Since acridine orange is a weak base, it will accumulate in an acid compartment. The acridine orange concentration is directly proportional to the acidity of the compartment. Microsomes preincubated with acridine orange at pH 6.0 and subsequently incubated at pH 8.0 in order to generate a pH gradient accumulated 0.73 ± 0.03 nmol acridine orange per mg protein ($n = 4$), while microsomes preincubated with the dye at pH 8.0 and incubated at pH 8.0 accumulated 0.16 ± 0.03 nmol acridine orange per mg protein, thus indicating that it was possible to produce a large vesicular pH gradient.

After producing vesicular pH gradients as described above, we showed that amiloride-sensitive Na^+ transport into microsomes was stimulated by 1.5- to 10-fold when the internal pH was 6.0 and the external pH was 8.0 compared with microsomes having a pH of 8.0 on both sides of the membrane. Amiloride-sensitive Na^+ transport was inhibited by 50–100% when the internal pH was 8.0 and the external was 6.0 as compared with the gradient-free control. The data shown in Table I are representative of data obtained from five separate experiments. Amiloride sensitive Na^+ transport into the microsomes was always more sensitive to pH gradients than was the amiloride-insensitive Na^+ transport. Under conditions similar to these, we have previously shown that there is no change in vesicular volume accompanying Na^+ uptake [1].

These data indicated that it was possible to generate a H^+ gradient across the vesicular membrane and that the direction and magnitude of the gradient could affect amiloride-sensitive Na^+ uptake. Therefore, it seemed possible that the amiloride-sensitive Na^+ transport into the microsomes was Na^+-H^+ exchange.

To verify further that Na^+-H^+ exchange was taking place in the vesicles, we measured H^+ movement in the presence of Na^+ gradients. When microsomes were preincubated with acridine orange at pH 6.0 and incubated for 2 min in the absence of Na^+, the amount of dye accumulated was found to be 2.35 ± 0.12 nmol/mg protein ($n = 3$). Microsomes preincubated with dye under the same conditions and incubated with sodium methanesulfonate (13.6 mM) were shown to accumulate only 0.87 ± 0.13 nmol dye per mg protein ($n = 3$). Therefore, Na^+ was capable of excluding acridine orange from the microsomes probably by driving protons out of the vesicles via Na^+-H^+ exchange and establishing a pH gradient. Indeed

TABLE I

EFFECT OF pH GRADIENT ON SODIUM TRANSPORT INTO TOAD BLADDER MICROSOMES

Aliquots (20 μl, 0.077 mg protein) of toad bladder microsomes were preincubated for 40 min at 20°C in a total volume of 40 μl with either pH 6.0 buffer (45 mM Mes/14 mM Tris/7 mM Hepes/180 mM sucrose) or pH 8.0 buffer (22 mM Tris/25 mM Hepps/202 mM sucrose) and then diluted with solutions (0.21 ml) containing ^{22}Na-sodium phosphate (2 μCi, 1.9 mM) and ouabain (0.1 mM), and either amiloride HCl (0.71 mM) or NaCl (0.71 mM) substituted for sodium phosphate and either pH 8.0 buffer (26 mM Tris/28 mM Hepps/195 mM sucrose/) or pH 6.0 buffer (52 mM Mes/8 mM Hepes/16 mM Tris/173 mM sucrose) and then incubated for 2 min at 20°C and applied to Dowex columns and eluted.

Preincubation pH (internal pH)	Incubation pH (external pH)	Na^+ uptake, mean ± S.D. ($n = 2$) (nmol/min mg protein)		
		− Amiloride	+ Amiloride	Difference
6.0	8.0	4.92 ± 0.25	2.05 ± 0.38	2.87
8.0	8.0	2.31 ± 0.07	1.59 ± 0.09	0.72
8.0	6.0	1.08 ± 0.36	0.74 ± 0.02	0.34

this proton gradient may limit Na^+ uptake since in vesicles at constant potential (held with valinomycin and K^+), addition of a proton uncoupler, 1799, stimulated Na^+ uptake by a factor of 1.2. This Na-dependent exclusion of acridine orange from the microsomes was inhibited 45% by amiloride (5 mM). Amiloride was also added to standard acridine orange solutions and shown to be incapable of quenching the fluorescence of acridine orange (data not shown). The failure of amiloride to block no more than half of the H^+ efflux from the microsomes might be due to several factors: first, the microsomes may be permeable to H^+. However, on the basis of the acridine orange accumulation, the microsomes can maintain a gradient of at least one pH unit for the duration of the incubation and assay period (about 2.5 min). A more likely possibility is that amiloride is not a very potent blocker of Na^+-H^+ exchange under our experimental conditions. The dose-response relationship for amiloride vs. Na^+ uptake in our microsomes is not saturated at 5 mM but amiloride is, unfortunately, insoluble at higher concentrations.

So far we have shown that there are correlated movements of Na^+ and H^+. The correlated movements might come about because of electrical potential gradients generated by movements of H^+ or Na^+. To eliminate this possibility we measured Na^+ uptake in the presence of valinomycin and different K^+ concentrations. Intravesicular K^+ is about 1 mM. Extravesicular K^+ from 0 mM (vesicle interior negative) to 4.75 mM (vesicular interior positive) in the presence of valinomycin (10 mg/l) had no effect on Na^+ uptake. Thus the amiloride-sensitive Na^+ uptake process is not sensitive to potential.

Besides an electroneutral Na^+-H^+ exchange system, an electroneutral Na^+/HCO_3^- co-transport system might have explained our results [13]. However, the uptake of $^{22}Na^+$ into toad bladder microsomes was shown to be insensitive to bumetanide (0.1 mM), a blocker of several Na^+/HCO_3^- transport systems [14] either in the presence or absence of amiloride when measured in the presence of a pH gradient (low pH inside) (see Fig. 1). Sodium transport into the microsomes was also shown to be totally insensitive to another HCO_3^- transport blocker, the stilbene derivative,

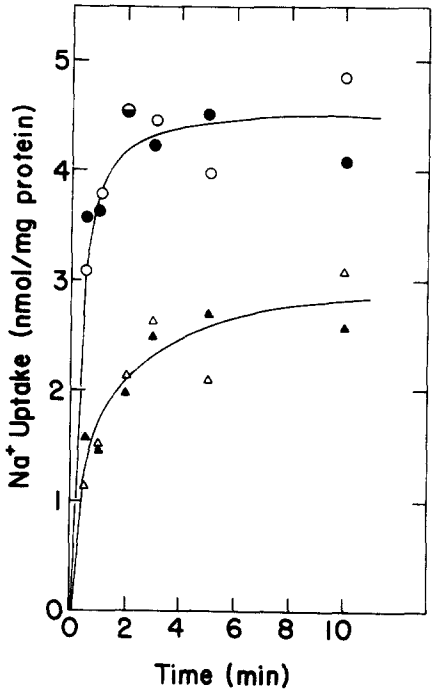

Fig. 1. Effect of amiloride and bumetanide on Na^+ influx into toad bladder microsomes. Sodium transport into toad bladder microsomes was measured at pH 8.0 as described in the legend of Table I after preincubation at pH 6.0, both in the presence of amiloride (0.6 mM) (Δ), bumetanide (0.1 mM) (●), both inhibitors together (▲), or neither inhibitor (○).

SITS (0.1 mM) [15] either in the presence or absence of a pH gradient across the microsomal membranes (data not shown). Since neither compound inhibited Na^+ transport into microsomes, we concluded that neither Na^+/HCO_3^- co-transport nor Cl^--HCO_3 exchange processes were active in the microsomes and that the uptake of Na^+ into the microsomes represented simple Na^+-H^+ exchange.

This work demonstrates another example of the growing list of tissues which display Na^+-H^+ exchange. The demonstration in this tissue is interesting from a comparative standpoint because of the large body of electrophysiological data available on the whole tissue.

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